Research in Biotechnology, 5(6): 24-32, 2014 ISSN: 2229-791X
www.researchinbiotechnology.com

Regular Article

Isolation and Characterization of some intracellular pigmented bacteria isolated from Soil & Coal powder

Debasis Mitra[1], Arup Kumar Mondal[2], Saikat Acharya[2], Arijit Mukhopadhyay*[1]

[1]Plant Tissue Culture Lab., Department of Biotechnology, Oriental Institute of Science & Technology, VIH campus, Rangamati, Paschim Medinipur, West Bengal- 721102 India
[2]Department of Biotechnology, Vidyasagar University, Rangamati, Paschim Medinipur, West Bengal- 721102 India
*Corresponding author E-mail: aribak@rediffmail.com

We have isolated some intracellular pigmented (Red, yellow, pink and orange colour colony) bacteria from West Bengal and Odisha state. It was screened for biochemical test and pigment production, protease activity and gelatin liquidation works. In this study, two types of bacteria (AD2 & SOM3) isolated from coal powder and other two types of bacteria (ADA4 & DM5) isolated from soil sample using serial dilution method. Other study focused that; all bacterial strain growth on temperature was also looked into the different condition. Colony formation allows to determine the number of bacteria in NAM, the cell density in the all bacterial strain is count. ADA4 has highly protease activity see in CMC agar and Skimmed milk agar.Hence microbial pigment production is one of the emerging fields of research to demonstrate its potential for various industrial applications and Bio-research works.

Keywords: Pigment production, protease activity, coal powder, cell density.

Colour is a vital constituent and it is probably one of the first characteristics perceived by the senses. Natural colours are generally extracted from fruits, vegetables, roots and microorganisms and are often called "biocolours" because of their biological origin (Pattnaik *et al.,* 1997). Natural pigments can be obtained from two major sources, plants (Mizukami *et al.,*1978) and microorganisms (Ryu *et al.,* 1989). Microbial pigments are of industrial interest because they are often more stable and soluble than those from plant or animal sources (Gunasekaran *et al.,*2008). Microorganisms can grow rapidly which can lead to high productivity and can produce a product throughout the year (Jiang *et al.,* 2005). Microorganisms have been used for a long time for production of molecules like antibiotics, enzymes, vitamins, texturizing agents and so on. There is a growing interest in the food industry in the use of natural ingredients (Falk *et al.,* 1983). Ingredients such as colorants are considered natural when derived from biological sources like plants or microorganisms. Some natural colorants have commercial potential for use as antioxidants. The microbial pigments are produced for applications in food, cosmetics or textiles Yusof (2006). Microorganisms produce various pigments like carotenoids, melanin, flavones, quinines, and more specifically monascins, violacein or indigo describe byDufosse (2006). Which are different pigment producing bacteria, *Staphylococcus aureus* Produces Golden yellow pigment, *Serratia*

marcescens produces red pigment, *Micrococcus lutes* produce yellow, *Micrococcus roseus* produce pink, *Staphylococcus roseus*produce red, and *Pseudomonas cynzatha* produces yellow pigment (Ahmad *et al.,* 2012; Harley, 2007). Bacteria produce pigments for various reasons and it plays an important role. Some bacteria such as cyanobacteria have phycobilin pigments to carry out photosynthesis describe by MacFaddin (1980). Carotenoids are a group of bioactive compounds and that's are responsible for bright yellow, orange, red pigments of various plants, microorganisms and animals (Wilhelm *et al.,* 1996) and are widely distributed in nature (Goodwin *et al.*,1988). These pigments have an important function to act as protective agents against oxidative damage (Scolnik *et al.*,1995). Recently carotenoids have attracted greater attention due to the beneficial role on human health. Carotenoids can inhibit various types of cancer and it enhanced the immune response (Krinsky *et al.,* 2005). It also protects "life style related" diseases such as cardiovascular disease and age related macular degeneration due to their antioxidant activity and pro vitamin A function (Young *et al.,* 2001).

Materials and Methods

Isolation and of bacteria

AD2 and SOM3: The bacterial strain was isolated from coal of Nabarun, Murshidabad, West Bengal-742236 India (Latitude-24.7815547; Longitude-87.9110672). The sample was serially diluted at 10^{-5} and dilution samples were plated onto nutrient agar medium. After 24 hours, we see that (AD2-Redish and SOM3-yellow) colony appeared. Pure culture was obtained by streaking 6 times in King's B medium and nutrient agar medium. Then the preservation for future we added 20% glycerol in pure culture and store at -20^{0}C Dubey (2007). The samples were incubated for 48 h at 37^{o}C with vigorous shaking at 150 rpm, to provide aeration for the bacteria

ADA4 and DM5: The sample was collected from soil of Puri, Baliopanda-752001 Odisha (Latitude-19.8133822; Longitude-85.8314654).The soil sample was serially diluted at 10^{-6} and 100µl dilution samples were plated onto nutrient agar medium. After 48hours, we see that (ADA4-orange, DM5-pink) colony appeared. Pure culture was obtained by streaking 4 times in King's B medium. Then the preservation for future, are same procedure following AD2 and SOM3.

Biochemical characteristics

Indol Test

Indole test is used to determine the ability of an organism to spilt amino acid tryptophan to form the compound indoledescribe by Dubey (2007).Inoculate the test bacteria in tryptophan broth with broth culture or emulsify isolated colony of the test organism in tryptophan broth. Incubate at 37°C for 28 hours in ambient air. Add 0.5 ml of Kovac's reagent to the broth culture (MacFaddin, 1980).Positive: Pink colored rink after addition of appropriate reagent and Negative: No color change even after the addition of appropriate reagent.

MR-VP

Lightly inoculate the tube from a single colony, preferably 24 hour culture. Slightly loosen the cap and incubate the tubes at 28°C for 48 hours. After incubation, use a sterile pipette to remove 1mL aliquots and place into two small tubes describe by Aneja (2003). One tube is for the methyl red test and the other for the Voges-Proskauer test. Add 5 drops of methyl red to one tube. Read the result immediately. For the Voges-Proskauer test add 15 drops of Voges-Proskauer a reagent (Mesaros *et al.,* 2007). Mix well to aerate the sample. Add 5 drops of Voges-Proskauer B to the tube and mix well to aerate the sample. Read the results within 5 minutes. M-R: A red color at the surface is considered a positive result. A negative test is indicated by a yellow color at the surface. V-P: A positive test is

indicated by a pink-red color developing within 5 minutes.

Catalase activity

The purpose is to see if the microbe has catalase, a protective enzyme capable of destroying the dangerous chemical hydrogen peroxide. Growth from an overnight culture of the microbe is smeared on a microscope slide (Aneja, 2003). A drop of 3% hydrogen peroxide is added (Rollins, 2009). If copious bubbles are observed, the microbe is positive for catalase.

Hydrogen sulfide production

After degradation Hydrogen sulfide, this can be easily detected on Kligler's agar by formation a black precipitate at the stabbing side. It can be examined by using the P^H indicator phenol red in the medium (Aneja, 2003).

Phenylalanine Deaminase test

An inoculum from a pure culture is transferred aseptically to a sterile tube of phenylalanine agar to streak the slant. The butt of the tube need not be stabbed (Raducanescu *et al.*,1986). The inoculated tube is incubated at 37^0C for 24 hours. After incubation, five drops of 10% ferric chloride and five drops of 0.1N HCl are added and the tube is gently shaken (Buiuc *et al.,* 2009). A positive result is indicated if a green color develops within five minutes.

Casein Hydrolysis

Casease is an exo-enzyme that is produced by some bacteria in order to degrade casein. Casein is a large protein that is responsible for the white color of milk (Ramette*et al.*, 2003). This test is conducted on milk agar which is a complex media containing casein, peptone and beef extract (Brown *et al.*, 1970). Those bacteria can produce casein, and then there will be a zone of clearing around the bacterial growth.

Urease Test

The urease test identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. It is primarily used to distinguish urease-positive. Prepare this UTM medium (Peptone-1gm, Dextrose-1gm, Sodium chloride-5gm, Potassium phosphate, monobas-2gm, Urea-20gm, Phenol red-0.012gm, Agar-15gm, dH_2O-1lit.) and added 100µl bacterial culture Dubey (2007). After one day results are appeared.

Gelatin hydrolysis

Gelatin hydrolysis test is used to detect the ability of an organism to produce gelatinase (proteolytic enzyme) that liquefy gelatin describe by Harley& Klein (2007). Hydrolysis of gelatin indicates the presence of gelatinases (Aneja, 2003). Inoculate a heavy inoculum of test bacteria (24 hour old) by stabbing 5 times on the tube containing nutrient gelatin medium (Chen *et al.*, 2006). Incubate the inoculated tube along with an inoculated medium at 35°C, or at the test bacterium's optimal growth temperature, for up to 2 weeks (Leboffe *et al.*, 2010). Remove the tubes daily from the incubator and place in refrigerator (4^oC) for 30 minutes (until control is gelled) every day to check for gelatin liquefaction (Madigan et al., 2012; McDade *et al.,* 1959). Positive: Partial or total liquefaction of the inoculated tube (inoculated control medium must be completely solidified) even after exposure to cold temperature of ice bath or refrigerator (4^oC), Negative: Complete solidification of the inoculated tube even after exposure to cold temperature of ice bath or refrigerator (4^oC).

Bacterial growth under stress temperature (Tm)

To determine the effect of temperature (T_m) (0-60^0C), the extent of growth of the isolate was determined by measuring the $O.D._{660}$ of spent culture at the aforementioned conditions.

Bacterial growth under high pH

The ability of the strain to adapt to pH, stress may be important for the several of the bacteria during drought and different physiological condition. In order to find out the optimal condition for growth of the test

bacteria, the effect of pH levels on the development was examined (Mitra *et al.*,2014)

Cell density

A bacterium growing on an agar surface also divides. Since most bacteria are not very motile on a solid surface, the progeny bacteria remain very near the location of the original bacterium. Colony formation allows one to determine the number of bacteria in a culture describe by Freifelder (2006).

Pigment production

AD2, SOM3, ADA4 and DM5 strain were grow in various culture medium. For confirm fresh sample were raised from glycerol stock and incubated for 48 hours at 28^0C with shaking at 250 rpm. A loopful of resulting bacterial culture suspension was streaked onto NAM and King's B medium. After 48 hours, take resulting (colony colour, size) of bacterial colony.

Results and Discussion

Morphology of the isolated strains

After the complete of staining procedure, see (40X & 100X) under microscope, that four bacterial strains were different types of gram nature and colony shape. Results are shown in Table 1.

Table 1 Morphological, pigment production of the isolated strain

Sl. No.	*Bacteria Name*	*Colour of Colony*	*Gram-Nature*	*Morphology*
1.	AD2	Red	+ve	Rod
2.	SOM3	Yellow	+ve	Small rod
3.	ADA4	Orange	–ve	Rod
4.	DM5	Pink	–ve	Small

Legend: [+ve]: gram positive;[-ve]: gram negative

Biochemical characterizations of the isolated strain

Biochemical test in order to determine the physiology of the isolated strain, a series of biochemical tests were performed (Tab-2& 4).

Growths under different temperature and pH

The ability of the bacterial strain to adapt to temperature, stress may be important. In this condition, inoculated four bacterial test samples for 48 hours, then take the resulting data at 660 nm in UV/Visible Spectrophotometer (Fig. 1 to 4: growth under temperature; Fig. 2 to 8: growth under pH).

Table 2. Biochemical Test

S. No.	*Bacterial strain*	Indol	MR	VP	Oxidase	Citrate	H_2S production	Ammonia productio	Starch hydrolysis	Casein hydrolysis	Urease	IAA	catalase	CMC	Gelatin liquation	Phe-ala
1.	**AD2**	+	+	-	+	+	-	+	+	-	+	nd	nd	nd	nd	+
2.	**SOM3**	+	+	-	-	+	±	+	-	-	+	nd	nd	nd	±	+
3.	**ADA4**	+	-	-	+	±	-	+	+	+	-	nd	+	+	+	+
4.	**DM5**	+	+	+	+	nd	-	+	+	+	+	+	nd	+	+	+

Legend: +: positive; -: negative; ±: variation results; nd: no data

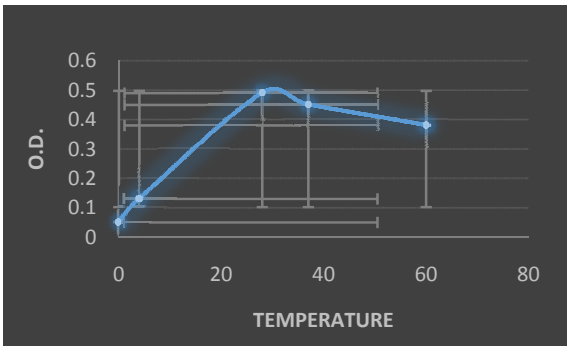

Figure 1. Growth under T_m by AD2

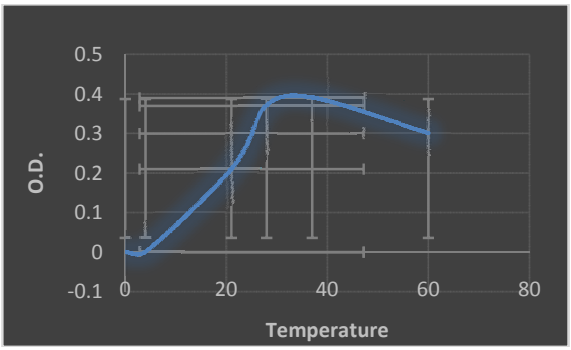

Figure 2. ADA4 growth under T_m

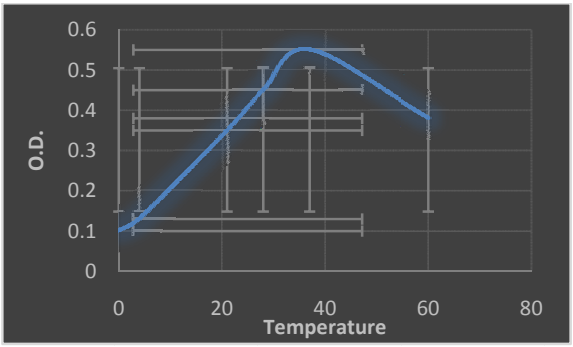

Figure 3. Growth under T_m performed by DM5

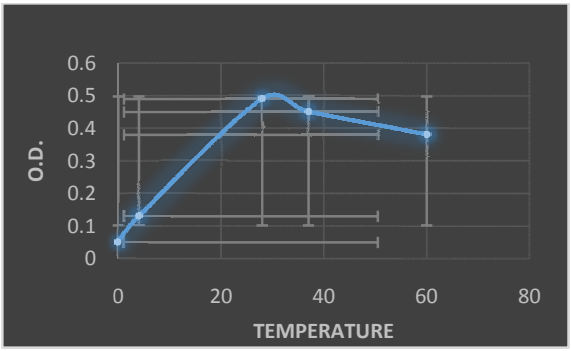

Figure 4. SOM3 growth under T_m

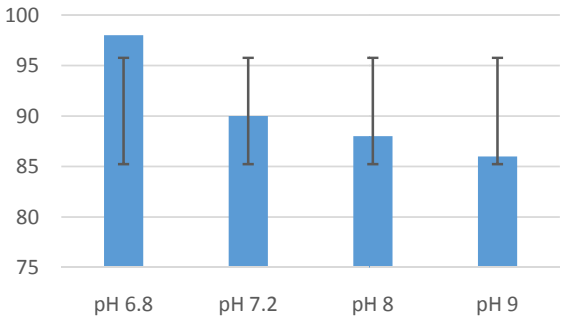

Figure 5. AD2 growth in high pH

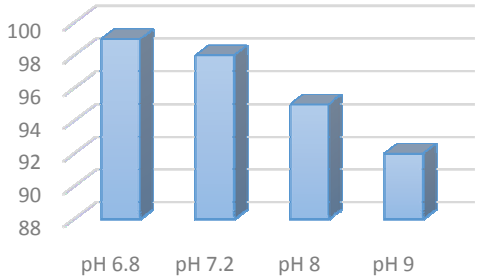

Figure 6. Growth under pH by SOM3

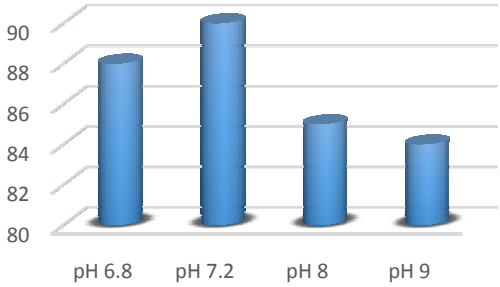

Figure 7. ADA4 growth in stress condition

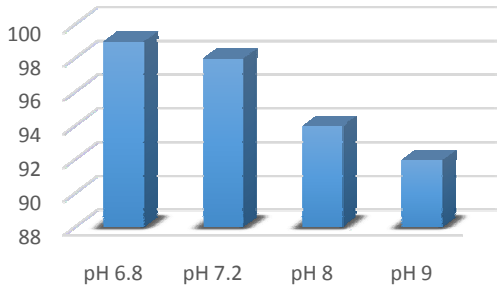

Figure 8. DM5 growth under pH

Pigment production

All bacterial strain was re-culturing in nutrient agar medium at 37^{0}C. After 24 hrs, SOM4 and DM5 strain were clearly developed yellow and pink pigment colony. Next 48 hrs, we see ADA4 and AD2 are developed orange and red pigment colony. Results are shown in Table 1 & 3 and fig 9.

Table 3 Bacterial growth rate and pigment production rate

Sl. No.	*Bacterial name*	*Growth Rate*		*Pigment*	*production*
		King's B	**NAM**	**Colour formation**	**Zone**
1.	**AD2**	**+**	**+++**	**++**	**++**
2.	**SOM3**	**+++**	**+++**	**+++**	**+++**
3.	**ADA4**	**+**	**++**	**++**	**++**
4.	**DM5**	**+++**	**+++**	**+++**	**+++**

Legend: +++: High, ++: Medium, +: Low

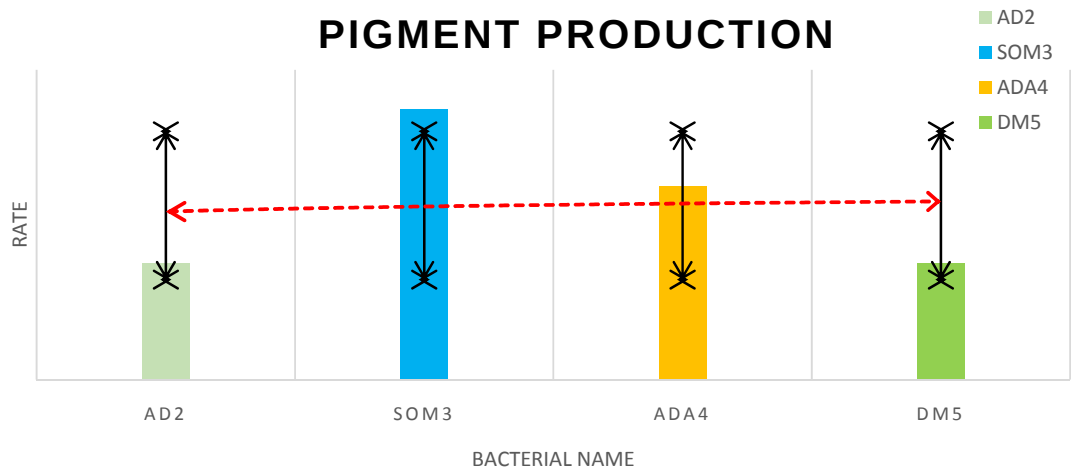

Figure 9. Pigment production rate

Protease activity

Protease assays are widely used for the investigation of protease inhibitors and detection of protease activities. The method is based on the speed at which the enzyme hydrolyzes ethyl lactate 30% (v/v) at pH 6.8 (Aneja, 2003). The lactic acid which is formed is titrated with sodium hydroxide and the consumption of the latter recorded as a function of time. In this study ADA4 bacterial strain has highly protease activity as following the results (Tab-2).

Cell density of the isolated strains

Colony formation allows to determine the number of bacteria in a culture. Then 0.6mL of 10^{-6} fold dilution of the culture is plated. Result are shown in Tab-5.

Table 4 Enzyme Production

Sl. No.	**Bacterial name**	**Cellulose**	**Protease**
1.	ADA4	1.00±0.05	1.10±0.05
2.	DM5	1.10±0.03	1.20±0.05

Table 5 Cell density

Sl. no.	*Strain name*	*Cells (cells/ml)*
1.	AD2	1.5×10^8
2.	SOM3	2×10^9
3.	ADA4	1.2×10^8
4.	DM5	2×10^9

Conclusion

The present study was an attempt to demonstrate the multifunctional property of this strain. This report highlights the key role of bacterial pigmentation, growth on pH &temperature and cell density. We see that DM5, ADA4 and SOM3 has different unique performing property. All strain, ability, one of the emerging fields of various biological research and industrial application.

Acknowledgements

We are very happy to take this opportunity to express our gratitude to Dr. (Mrs) T. Mandal, Principal, Department of Biotechnology, Oriental Institute of Science & Technology, Vidyasagar University, VIH campus, Rangamati, Paschim Medinipur, West Bengal- 721102 India and Board of Management of Oriental Institute of Science and Technology, West Bengal, India. The acknowledgement cannot end without mentioning the constant support rendered by our family members.

References

Ahmad WA, Wan Ahmad WY, Zakaria, ZA, Yusof NZ. 2012. Application of Bacterial Pigments as Colorant. VIII, 77p. 50 illus., 15 illus. in color.

Anija, KR. 2003. Exp. In microbiology, plant pathology and biotechnology, fourth ed., ISBN: 81-224-1494-X.

Brown MR, Foster JH. 1970. A simple diagnostic milk medium for *Pseudomonas aeruginosa*. J Clin. Pathol. Mar; 23(2):172–177.

Buiuc D, Marian N. 2009. Tratat de microbiologic clinica. Ed. III. 1138.

Chen YP, Rekha PD, Arun AB, Shen FT, Lai WA, Young CC. 2006. Phosphate solubilizing bacteria from sub-tropical soil and their tri calcium phosphate solubilizing abilities. Applied soil ecology, 34; 33-41.

Dubey, RC and Maheshwari DK. 2007. Prac. Microbiology, sec. ed. ISBN: 81-219-21538.

Dufosse L. 2006. Microbial production of food grade pigments. J. Food Techn. Biotechn, 44(3): 313-321.

Falk D, and Guering SJ. 1983. Differentiation of *Staphylococcus* and *Micrococcus spp*. with the Taxo A bacitracin disc. J. Clin. Microbiol., 18(3): 719–721.

Freifelder D. 2006. Molecular biology. 2F-2G 53 Syed Amir Ali avenue, Calcutta 700019. 2nd edition.ISBN:81-85198-34-9.

Goodwin TW, Britton.1988. Distribution and analysis of carotenoids in plant pigments. Acad Press London United Kingdom: 61-132.

Gunasekaran S, Poorniammal R. 2008. Optimization of fermentation conditions for red pigment production from *Penicillium sp*. under submerged cultivation. Afr. J. Biotechn., 7(12): 1894-1898.

Harley, JP, Prescott, LM, and Klein, DK. Microbiology 7th ed. Mcgraw-hill, NewYork .2007; ISBN-978-0-07-110231-5.

Harley JP. Prescott LM. 2007. Laboratory exercises in microbiology. 7th Edition. McGraw- Hill.

Jiang Y, Chen F, and Hyde KD. 2005. Production potential of water-soluble Monascus red pigment by a newly isolated *Penicillium sp*. J. Agr. Techn., 1(1):113-126.

Krinsky NI, Johnson EJ .2005. Carotenoid actions and their relation to health and disease. Mol Aspects Med 26: 459-516.

Leboffe MJ, Pierce BE. 2010. Microbiology laboratory theory and application, 3rd ed. Morton Publishing Company, Englewood, CO.

MacFaddin J. 1980. Biochemical tests for identification of medical bacteria, 2nd edn. Williams and Wilkins, Baltimore.

MacFaddin, Jean F. Biochemical Tests for Identification of Medical Bacteria. Williams & Wilkins, 1980, pp 173 – 183.

Madigan MT, Martinko JM, Stahl DA, Clark DP. 2012. Brock biology of microorganisms, 13th ed. Benjamin Cummings, San Francisco, CA.

McDade JJ, Weaver RH. 1959. Rapid methods for the detection of gelatin hydrolysis. J. Bacteriol. 77:60–64.

Mesaros N, Nordmann P, Ple´siat P, Roussel-Delvallez M, Van Eldere J, Glupczynski Y, Van Laethem Y, Jacobs F, Lebesque. 2007. *Pseudomonas aeruginosa*: resistance and therapeutic options at the turn of the new millenium. Clin Microbiol Infect 13:560–578.

Mitra D, Mondal, KA, Mukhopadhyay, A. 2014. Unique level of Multi-Drug Resistance (MDR) in *Pseudomonas aeruginosa strain* DEB1. Int. J. Sci Res 3 (9), 455-460.

Mizukami H., Konoshima M. and Tabata M. 1978 Variation in pigment production in *Lithospermum erythrorhizon* cultures. Phytochem., 17:95-97.

Pattnaik P, Roy U, and Jain P. 1997. Biocolours: New Generation Additives for Food, Indian Food Industry, 16(5): 21-32.

Raducanescu H, Bica-Pupil V. 1986. Bacteriologic veterinara; ed. Cerea. Bucuresti.

Ramette AM, Frapolli G, Defago Y. 2003. Mol. Plant microbe in., 16, 525.

Rollins DM (2000-08-01). Bacterial Pathogen List. BSCI-424 Pathogenic Microbiology. University of Maryland. 2009-03-01.

Ryu BH, Park BG, Chi YE, Lee JH. 1989. Production of purplish-red pigment in mixed culture of *Streptomyces propurpuratus* and *Bacillus sp*. Korean J. Applied Microbiol. Bioeng., 17:327-333.

Scolnik PA, Bartley GE 1995. Nucleotide sequence of a putative geranyl geranyl pyrophosphate synthase from Arabidopsis. Plant Physiol 104: 1469-1470.

Wilhelm S, Helmut S. 1996. Lycopene: A biologically important carotenoid for humans? Archives of Biochemistry and biophysics 336: 1-9.

Young AJ, Lowe GM. 2001. Antioxidant and prooxidant properties of carotenoids. Archives of Biochemistry and biophysics 385: 20-27.

Yusof NZ. 2006. Isolation, characterizations and application of pigments from coloured bacteria. *Dissertation*, University of Malaysia.